

## DOES PHEROMONE BIOLOGY OF *Lambdina athasaria* AND *L. pellucidaria* CONTRIBUTE TO THEIR REPRODUCTIVE ISOLATION?

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**Abstract**—Recently, 7-methylheptadecane and 7,11-dimethylheptadecane have been reported as sex pheromone components of both spring hemlock looper (SHL), *Lambdina athasaria*, and pitch pine looper (PPL), *Lambdina pellucidaria*. Our objective was to test the hypothesis that SHL and PPL are reproductively isolated, in part, through species specificity in: (1) absolute configuration of pheromone components, (2) diel periodicity of pheromonal communication, and/or (3) seasonal flight period. In coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses of stereoselectively synthesized (7*S*)- and (7*R*)-7-methylheptadecane [7*S*; 7*R*]

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as well as (7*S*,11*S*)-, (7*R*,11*R*)-, and (*meso*-7,11)-7,11-dimethylheptadecane [7*S*,11*S*; 7*R*,11*R*; *meso*-7,11], only 7*S* and *meso*-7,11 elicited responses by male SHL and PPL antennae. In field experiments, male SHL and PPL were attracted only to lures containing 7*S* plus *meso*-7,11. In hourly recordings of trap-captured males, SHL and PPL in their respective habitats were trapped between 24:00 and 03:00 hr. Capture of both SHL and PPL in pheromone-baited traps throughout June indicated overlapping seasonal flight periods. These findings of identical absolute configuration of pheromonal components, diel periodicity of pheromonal communication, and overlap of seasonal flight periods support synonymy of SHL and PPL. Finite taxonomic classification of PPL and SHL must await careful assessment of further criteria, such as morphometrics, molecular comparisons and ecological analyses.

**Key Words**—Geometridae, 7-methylheptadecane, 7,11-dimethylheptadecane, pheromone chirality, diel periodicity of pheromonal communication, seasonality of flight, reproductive isolating mechanisms, synonymy.

## INTRODUCTION

The spring hemlock looper (SHL), *Lambdina athasaria*<sup>7</sup> (Walker), and pitch pine looper (PPL), *L. pellucidaria* (Grote and Robinson), have remarkably similar life histories (summarized in Duff, 1998). Although they prefer hemlock (SHL) and pine (PPL) forests, they are neither spatially nor temporally completely isolated. Both SHL and PPL overwinter as pupae in the forest duff. Adults begin to emerge in mid to late May and are present until the end of June or early July. With identical sex pheromone blends (7-methylheptadecane and 7,11-dimethylheptadecane) identified in both species (Gries et al., 1994; Maier et al., 1998), alternative mechanisms of reproductive isolation needed to be investigated. Here we tested the hypotheses that PPL and SHL are reproductively isolated, in part, through species specificity in: (1) absolute configuration of pheromone components; (2) diel periodicity of pheromonal communication; and/or (3) seasonal flight period.

## METHODS AND MATERIALS

**Syntheses.** (7*S*)-7-Methylheptadecane, (7*R*)-7-methylheptadecane, (7*S*, 11*S*)-7,11-dimethylheptadecane, (7*R*,11*R*)-7,11-dimethylheptadecane and (*meso*-7,11)-7,11-dimethylheptadecane, hereafter referred to as 7*S*, 7*R*, 7*S*,11*S*, 7*R*,11*R*, and *meso*-7,11 (Figure 1), were synthesized as previously described (Sheng,

<sup>7</sup>Univoltine *L. athasaria* investigated in this study is not to be confused with the bivoltine oak feeder, *L. fervidaria athasaria* (Walker), which is illustrated by Covell (1984). Larvae of *L. athasaria* considered here are restricted to eastern hemlock.

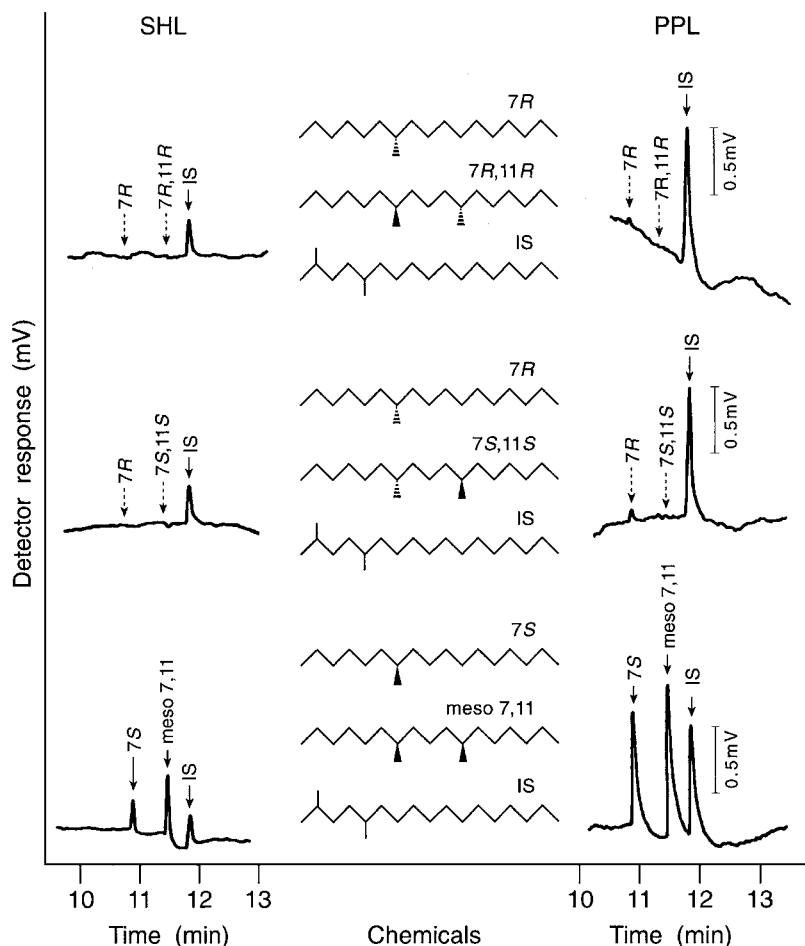


FIG. 1. Representative GC-EAD recordings from male spring hemlock looper (SHL) and from male pitch pine looper (PPL) antennae, responding to 1000 pg of 2,5-dimethylheptadecane [internal standard (IS)] and to 2 pg of optical isomers of pheromone components. Antennal recordings conducted in consecutive order (top to bottom), employing the same antenna. Flame ionization detector (FID) recordings omitted. Compound nomenclature: 7R = (7R)-7-methylheptadecane; 7S = (7S)-7-methylheptadecane; 7R,11R = (7R,11R)-7,11-dimethylheptadecane; 7S,11S = (7S,11S)-7,11-dimethylheptadecane; meso 7,11 = (meso-7,11)-7,11-dimethylheptadecane. Chromatography: Hewlett Packard 5890 equipped with a fused silica column (30 m  $\times$  0.25 mm ID) coated with DB-5 (J&W Scientific, Folsom, California); splitless injection, temperature of injection port and FID detector: 250°C; temperature program: 120° for 1 min, then 10°C/min to 280°C.

1996; Shirai, 1997; Shirai et al., 1999). Based on gas chromatographic analyses, all compounds used in field experiments were >94% chemically pure. Compounds were 99–100% enantiomerically pure as estimated by the enantiomeric excess (99.8–100%) of the starting material (enantiomers of methyl 3-hydroxy-2-methyl propanoate).

**Laboratory Analyses.** Adult male SHL and PPL were collected at night in Peoples State Forest, Barkhamsted, Litchfield County, Connecticut, and Myles Standish State Forest, Plymouth, Plymouth County, Massachusetts, respectively. Moths were cooled (8°C) overnight and couriered to Simon Fraser University the next day for coupled gas chromatographic–electroantennographic detection (GC-EAD) analyses (Arn et al., 1975). Each antenna tested (four and five for male SHL and PPL, respectively) was subjected in the same order to three mixtures of candidate pheromone components at 2 pg each. Mixtures 1–3 contained 7R plus 7R,11R, 7R plus 7S,11S, and 7S plus *meso*-7,11, respectively. An internal standard, 1 ng of 2,5-dimethylheptadecane, was coinjected with each mixture, to monitor possible deterioration of antennal responses over the 30–40 min test period for each antenna.

**Experimental Sites.** Experiments with SHL and PPL were conducted at Peoples State Forest and Myles Standish State Forest, respectively. In Peoples State Forest 120 km inland, the transition-zone forest of gymno- and angiosperm trees forms a dense canopy with hemlock, *Tsuga canadensis* (L.) Carr., as the dominant species. The forest lies at 300 m in elevation and receives 306 mm of rain from June to August (Owenby and Ezell, 1992). The pine forest at Myles Standish State Forest exhibits an open forest canopy with pitch pine, *Pinus rigida* Miller, as the dominant species. Directly on the Atlantic coast, the pine forest experiences a moderate, ocean-influenced climate, with little summer rain and prolonged periods of drought (Schweitzer and Rawinski, 1988). More detailed characteristics about experimental sites and their locations are provided in Duff (1998).

**General Design of Field Experiments.** Experiments of identical or compatible design, conducted concurrently for both SHL and PPL (except experiment 7), were set up in randomized, complete blocks with traps and blocks at 15- to 20-m intervals. Green Unitraps (Phero Tech Inc., Delta, British Columbia, Canada) were suspended from *T. canadensis* (SHL) and *P. rigida* (PPL) at 1.5–2 m above ground and 2–5 m within the forest margin. Traps were baited with grey rubber septa (The West Company, Lionville, Pennsylvania) impregnated with stereoisomers of synthetic pheromone components in HPLC-grade hexane. Each trap contained a dichlorvos cube (Bio-Strip, Inc., Reno, Nevada) to kill captured males. Moths were removed and counted every two days.

**Field Test of Optical Isomers of Pheromone Components.** Experiment 1 (SHL) and experiment 2 (PPL) tested stereoisomeric 7,11 alone and in combination with either 7S, 7R or both. The 7S (experiment 3, SHL) or 7S plus 7R

(experiment 4, PPL) were tested alone and in binary and quaternary combinations with 7S,11S, 7R,11R, and *meso*-7,11. Experiment 5 (SHL) tested 7S alone and in binary combination with *meso*-7,11 and all possible ternary and quaternary combinations with 7S,11S, 7R,11R, and *meso*-7,11. Experiment 6 (PPL) was identical to experiment 5, but replaced 7S with 7S + 7R. Experiment 7 was conducted only with PPL and tested 7S plus *meso*-7,11 alone and in combination with 7R at three different ratios. Experiment 8 (SHL) and experiment 9 (PPL) tested 7S alone and in combination with *meso*-7,11 at three different ratios. Experiment 10 (SHL) and experiment 11 (PPL) tested *meso*-7,11 alone and in combination with 7S at three different ratios. Experiment 12 (SHL) and experiment 13 (PPL) tested 7S plus *meso*-7,11 at increasing doses.

*Height-Dependent Trap Captures of Male SHL and PPL.* For experiment 14 and 15, ten trees >200 m apart were selected at each experimental site. Unitraps baited with grey rubber septa impregnated with 50  $\mu$ g 7S and 16  $\mu$ g *meso*-7,11 were hung at 1.5, 3, and 5 m above ground in each tree. Captured male SHL (experiment 14) or PPL (experiment 15) were recorded two days after experiment initiation.

*Diel Periodicity and Seasonality of Trap Captures.* Experiments 16 and 17 recorded the diel periodicity of captures of male SHL and PPL, respectively. Ten trees >100 m apart were selected at each experimental site. One Unitrap baited with grey rubber septa impregnated with 50  $\mu$ g 7S and 16  $\mu$ g *meso*-7,11 was hung 1.5 m above ground and 2–5 m within the forest margin in each tree. Captured males were recorded hourly for 24 hr. Experiments 18 and 19 recorded seasonal flight periods of male SHL and PPL, respectively. Selection of trees and trap placement were the same as in experiments 16 and 17. Traps were baited with grey rubber septa impregnated with 50  $\mu$ g racemic 7 and 16  $\mu$ g stereoisomeric 7, 11. Captured males were removed and counted every two days from May 28, to June 24, 1997.

*Statistical Analyses.* Despite transformation, most trap catch data were not normally distributed and were therefore subjected to nonparametric analyses of variance by ranks (Friedman's test) followed by comparison of means (Bonferroni test) (Zar, 1984; SAS/STAT 1988 user guide, release 6.03 edition, SAS Institute, Cary, North Carolina).

## RESULTS

In GC-EAD recordings, 7S, but not 7R, and *meso*-7,11, but not 7S,11S or 7R,11R, elicited responses from male SHL and PPL antennae (Figure 1). In field experiments 1 and 2, 7S in combination with stereoisomeric 7,11 attracted male SHL and PPL (Figure 2). For both species, 7R was inactive with 7,11, but doubled captures of male PPL when added to 7S plus 7,11 (Figure 2, experiment 2). For

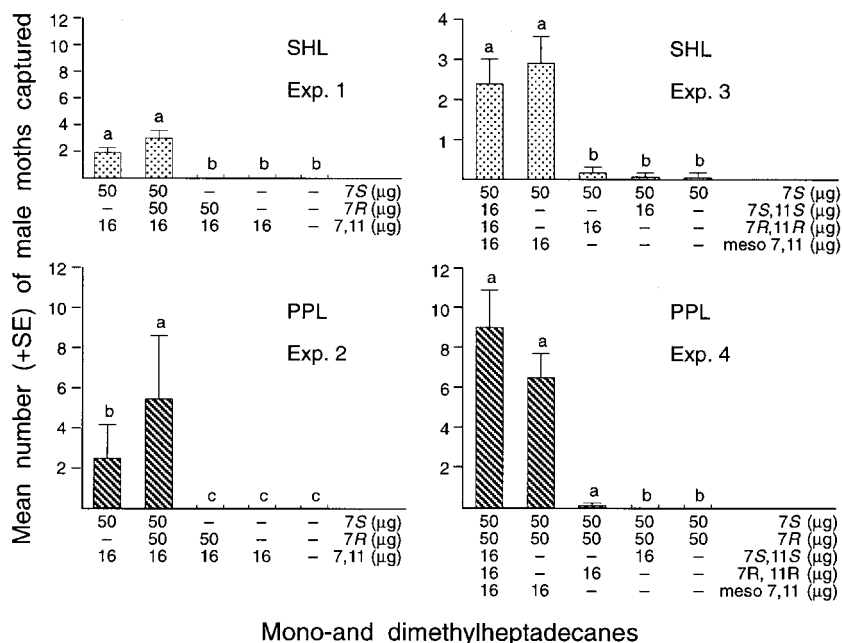
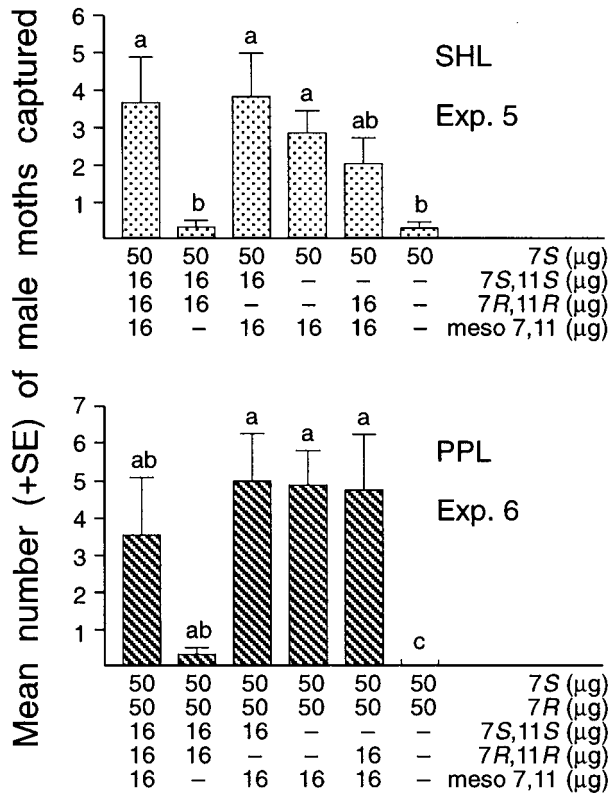


FIG. 2. Left: Captures of male spring hemlock loopers (SHL) (experiment 1; June 10–12, 1997; 10 replicates) and pitch pine loopers (PPL) (experiment 2; May 30–June 3, 1997; 10 replicates) in Unitraps baited with stereoisomeric dimethylheptadecane alone and in combination with 7*S*, 7*R*, or both. Right: Captures of male SHL (experiment 3; June 12–14, 1997; 10 replicates) and male PPL (experiment 4; June 3–5, 1997; 10 replicates) in Unitraps baited with 7*S* alone (SHL) or 7*S* plus 7*R* alone (PPL), and in binary and quaternary combinations with 7*S*, 11*S*, 7*R*, 11*R*, and *meso*-7,11. For each experiment, bars with the same letter are not significantly different,  $P > 0.05$ . Compound nomenclature as in Figure 1 legend. Note that when apparent behavioral activity of 7*R* in experiment 2 was retested in a subsequent experiment, it was inactive (Duff, 1998).

both SHL and PPL, the synergistic stereoisomer of 7,11 proved to be *meso*-7,11 (Figure 2, experiments 3, 4; Figure 3, experiments 5, 6); 7*S*, 11*S* and 7*R*, 11*R* were benign. Retesting 7*R* as a potential pheromone component for PPL in experiment 7 failed to disclose any behavioral activity associated with this compound (Duff, 1998). The blend of 7*S* plus *meso*-7,11 at a 50:16-μg ratio was more attractive to male SHL and PPL than blend ratios with reduced amounts of either *meso*-7,11 (Figure 4, experiments 8 and 9) or 7*S* (Figure 4, experiments 10 and 11). When 7*S* plus *meso*-7,11 were tested at five doses separated by orders of magnitude, only traps baited with the highest dose captured significant numbers of males (Figure 5, experiments 12 and 13).



**Mono- and dimethylheptadecanes**

FIG. 3. Captures of male spring hemlock loopers (SHL) (experiment 5; June 14–16, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 6; June 5–7, 1997; 10 replicates) in Unitraps baited with 7S alone (SHL) or 7S plus 7R alone (PPL), in binary combination with *meso*-7,11), and in all ternary and quaternary combinations with 7S,11S, 7R,11R, and *meso*-7,11. For each experiment, bars with the same letter are not significantly different,  $P > 0.05$ . Compound nomenclature as in Figure 1 legend.

Traps suspended at 1.5 m above ground captured significantly fewer male SHL than those at 5 m (Figure 6, experiment 14) and significantly fewer male PPL than those at 3 and 5 m (Figure 6, experiment 15). In hourly recordings of captured males, seven SHL and five PPL were captured between 24:00 hr and 0300 hr (Figure 6, experiments 16 and 17). The seasonal flight periods of SHL and PPL overlapped (Figure 6, experiments 18 and 19), but few SHL were captured until mid-June.

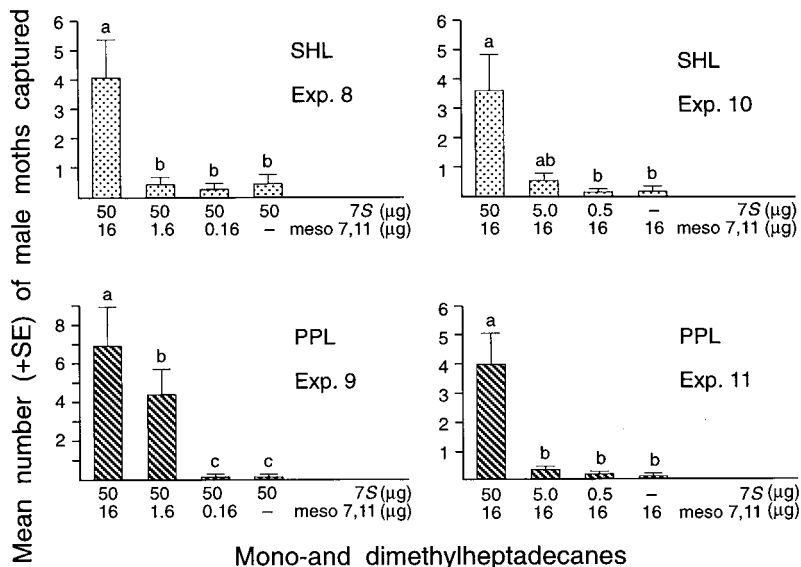


FIG. 4. Left: Captures of male spring hemlock loopers (SHL) (experiment 8; June 16–18, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 9; June 11–13, 1997; 10 replicates) in Unitraps baited with 7S alone and in combination with (*meso*-7,11) at three different ratios. Right: Captures of male SHL (experiment 10; June 18–20, 1997; 10 replicates) and male PPL (experiment 11; June 13–15, 1997; 10 replicates) in Unitraps baited with *meso*-7,11 alone and in combination with 7S at three different ratios. For each experiment, bars with the same letter superscript are not significantly different,  $P > 0.05$ . Compound nomenclature as in Figure 1 legend.

## DISCUSSION

Electrophysiological recordings (Figure 1) and field experiments (Figures 2–6) indicate that SHL and PPL lack specificity of their pheromone blends. Antennae from males of both species discriminated between optical isomers of pheromone components (mono- and dimethylheptadecanes), but invariably responded only to 7S and *meso*-7,11 (Figure 1). Attraction of male SHL and male PPL only to lures containing 7S + *meso*-7,11 (Figures 2–4) confirms enantio- and stereospecific recognition of pheromone components, and demonstrates that SHL and PPL use chiral pheromone components of identical absolute configuration. Because 7R, 7S,11S, or 7R,11R elicited neither antennal (Figure 1) nor behavioral responses (Figures 2–4), they can not be employed by female SHL or PPL to inhibit or reduce cross-attraction of heterospecific males. Moreover, different blend ratios of 7S + *meso*-7,11 had similar effects on captures of male



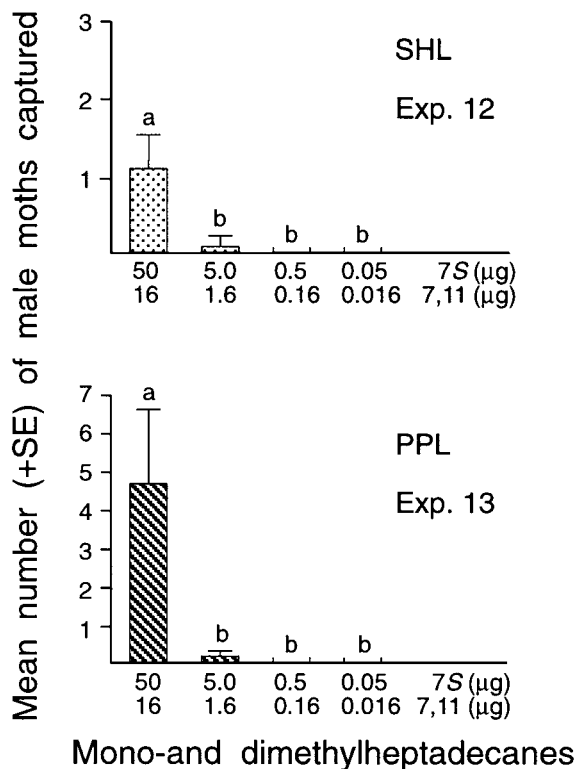


FIG. 5. Captures of male spring hemlock loopers (SHL) (experiment 12; June 20–22, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 13; June 15–17, 1997; 10 replicates) in Unitraps baited with 7S + *meso*-7,11 at four different doses. For each experiment, bars with the same letter are not significantly different,  $P > 0.05$ . Compound nomenclature as in Figure 1 legend.

SHL and PPL (Figure 4, experiments 8–11), eliminating blend ratios as a means of pheromonal specificity.

Enantiospecific perception of pheromone components by males implies enantioselective biosyntheses by females. Confirmation that female SHL and PPL stereoselectively produce 7S and *meso*-7,11, however, must await development of a chiral GC column that separates optical isomers of methyl- and dimethylheptadecane.

With identical sex pheromones, SHL and PPL could maintain reproductive isolation through temporal (diel and/or seasonal) separation of sexual communication. Sympatric and coseasonal artichoke plume moths, *Platyptilia carduidactyla* (Riley) and *P. williamsii* (Grinnell), for example, use identical sex

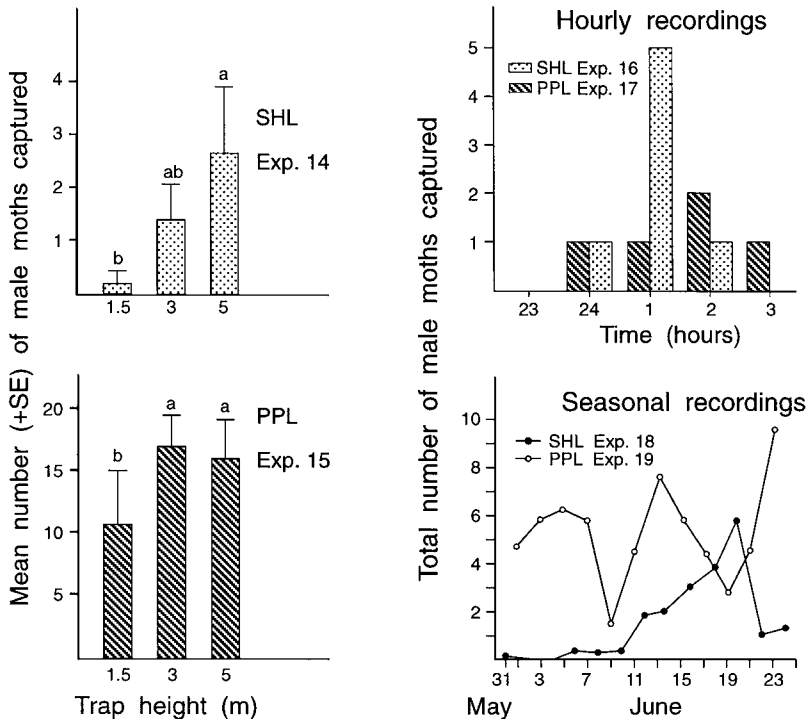


FIG. 6. Left: Captures of male spring hemlock loopers (SHL) (experiment 14; June 19–22, 1997; 10 replicates) and male pitch pine loopers (PPL) (experiment 15; June 17–21, 1997; 10 replicates) in Unitraps suspended from trees at 1.5, 3, and 5 m above ground and baited with 50  $\mu\text{g}$  7S plus 16  $\mu\text{g}$  *meso*-7,11. For each experiment, bars with the same letter are not significantly different,  $P > 0.05$ . Top right: Hourly recordings of trap captured male SHL (experiment 16; June 19–22, 1997; 10 replicates) and male PPL (experiment 17; June 17–18, 1997; 10 replicates) in Unitraps baited with 50  $\mu\text{g}$  7S plus 16  $\mu\text{g}$  *meso*-7,11. Bottom right: Seasonal recordings of trap captured male SHL (experiment 18; May 31–June 26, 1997; 10 replicates) and male PPL (experiment 19; May 31–June 26, 1997; 10 replicates) in Unitraps baited with enantiomeric 50  $\mu\text{g}$  7 and stereoisomeric 50  $\mu\text{g}$  7,11. Compound nomenclature as in Figure 1 legend.

pheromones but release them at different times of the night (Haynes and Birch, 1986). Diel periods of pheromone communication in SHL and PPL, in contrast, seem to overlap (Figure 6). Even though the seasonal flight period of SHL may commence ~7–10 days earlier than that of PPL (Maier and Lemmon, 1996), capture of male SHLs and PPLs in pheromone-baited traps throughout June (Figure 6) eliminates seasonality of flight as a reproductive isolating mechanism.

Spatial separation (between or within habitats) of sexual communication represents a third potential isolating mechanism. The SHL occurs in moist areas within forests of eastern hemlock. The PPL, in contrast, typically inhabits dry sandy areas with mainly pitch and other hard pines. While SHL and PPL are mostly allopatric in eastern North America (Maier and Lemmon, 1996), their habitats are not far apart and areas of sympatry may exist. Captures of male SHL and PPL mainly in traps suspended at 3 and 5 m (Figure 6) suggest that females of both species call in similar locations within the forest canopy, and that spatial separation of sexual communication would fail in coinhabited forests.

In summary, our study did not reveal discernible differences in the pheromone biology of PPL and SHL, supporting the contention that they should be synonymized (Zhang, 1994). Finite taxonomic classification of PPL and SHL, however, must await careful assessment of other criteria (Roelofs and Brown, 1982; Roelofs and Comeau, 1969), such as morphometrics, molecular comparisons, and ecological analyses.

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